# Sporulation and Germination Gene Expression Analysis of *Bacillus anthracis* Sterne Spores in Skim Milk under Heat and Different Intervention Techniques

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**ABSTRACT: To investigate how *B. anthracis* Sterne spores survive in milk under heat (80 °C, 10 min), pasteurization (72 °C, 15 s), microfiltration, and pasteurization and microfiltration, the expression levels of genes related to sporulation and germination were tested using real-time PCR assays. Twenty-seven sporulation- and germination-related genes were selected for the target genes. Our results demonstrated that gene expression levels were altered by heat and microfiltration whereas the pasteurization and pasteurization and microfiltration resulted in less alteration of gene expression. Heat activated and inhibited both sporulation- and germination-related genes, suggesting that bacterial spores underwent different molecular mechanism for heat treatments. Our results may provide some insight into the molecular mechanisms of spore survival in response to heat treatment and different intervention strategies used to treat fluid skim milk.**

**Keywords: *Bacillus anthracis* spores, milk pasteurization and microfiltration, real-time PCR assay**



## Introduction

The biohazardous attack with *Bacillus anthracis* spores in 2001 in the United States highlighted the importance of the biodefense system, especially for the food supply. Since *B. anthracis* spores could be deliberately released into the milk supply as a biohazardous agent (Wein and Liu 2005), research is needed to determine the behavior of spores in milk. *B. anthracis* spore inactivation in food, including milk, has been studied extensively (Hanson and others 2005; Xu and others 2006, 2008a, 2008b; Robertson and others 2008). Previous studies have shown that pasteurization (72 °C, 15 s) does not kill *B. anthracis* spores (Bowen and Turnbull 1992; Perdue and others 2003; Novak and others 2005) and may in fact stimulate the spores to germinate (Hanson and others 2005). Germinated spores could generate sufficient levels of enterotoxin to cause foodborne illness. In skim milk, the combination of microfiltration and pasteurization achieved a 5.6-log reduction in bacteria count (Elwell and Barbano 2006). However, the mechanism of bacterial spore survival remains unknown.

*B. anthracis* spores are extremely stable and can survive under very harsh conditions. *B. anthracis* spore germination involves the degradation of small acid soluble proteins and the release of dipicolinic acid (Nicholson and others 2000; Setlow 2006). *B. anthracis* sporulation and germination have been studied intensively, and a number of sporulation- and germination-related genes have been identified using transcriptional profiling (Bergman and others 2006). Recently, a transposon site hybridization (TraSH) assay was developed and 31 genes required for *B. anthacis* sporulation and germination were identified (Day and others 2007). However, there have been very few reports on how *B. anthracis* spores survive under different intervention techniques.

In this study, a real-time polymerase chain reaction (PCR) assay was used to study the expression levels of genes that are related to sporulation and germination following various processing techniques for fluid milk. *B. anthracis* spores were analyzed under heat treatment (80 °C for 10 min) in peptone water. Skim milk inoculated with *B. anthracis* Sterne spores was subsequently treated via pasteurization, microfiltration, and pasteurization and microfiltration to determine the expression or inhibition of targeted sporulation and/or germination genes, to potentially provide information for controlling *B. anthracis* spores that may be purposely added to milk.

## Materials and Methods

### Bacterial strain and spore preparations

Spores from the avirulent surrogate Sterne strain of *B. anthracis* were originally obtained from Drs. Jeff Karns and Michael Perdue (Perdue and others 2003; U.S. Dept. of Agriculture, Agricultural Research Service, Beltsville Area Research Center, Beltsville, Md., U.S.A.). A derivative strain was used herein. Spore preparations were performed essentially as described previously (Novak and others 2005).

### Sample treatments

**Heat treatment.** Heat treatment is often used to kill the residual vegetative cells in the spore preparation. The spores were heated to 80 °C for 10 min in sterile 0.1% peptone water.

**Milk pasteurization.** *B. anthracis* Sterne spores were placed in skim milk at a concentration of approximately $10^6$ spores/mL milk. The milk with *B. anthracis* spores was placed at 72 °C for 15 s.

**Milk pasteurization and microfiltration.** Raw skim milk was transferred aseptically to a sterile-stainless steel vessel and

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inoculated with the *B. anthracis* Sterne spores that were in sterile 0.1% peptone water to achieve a target level of approximately 6 $\log_{10}$ spores/mL of milk. After inoculation, milk was treated as follows: (i) microfiltered using a microfiltration Membralox Pilot Skid System (Pall Advanced Separation System, Cortland, N.Y., U.S.A.) with a ceramic membrane of nominal size (1.4 $\mu$m) at a cross flow velocity of 6 m/s (microfiltration), (ii) pasteurized at 72 °C for 15 s (pasteurization), or (iii) combined treatments of microfiltration (i) and pasteurization (ii). The retentates from the pasteurized or nonpasteurized milk obtained from accumulation of the spores, solids, and/or contaminants of the milk were aseptically collected in a sterile 50-mL screw-capped conical centrifuge tube after microfiltration of milk for 30 or 120 min.

All the experiments were performed in duplicate using spores +/− prior different treatments. Intervention component was conducted in the center of Excellence for Process Validation (CEPV) at the Eastern Regional Research Center (ERRC).

### Bacterial RNA isolation and cDNA synthesis

Bacterial RNA was isolated using the hot phenol method as described by Liu and others (2004) with the following modifications: bacterial spores resuspended in boiling lysis buffer were added to Zirconia Beads (Ambion, Tex., U.S.A.) and disrupted for 10 min using the mini-Beadbeater-8 (BioSpec Products Inc., Bartlesville, Okla., U.S.A.). RNA quantity and quality were assessed by measuring the ratio of absorbance at 260 and 280 nm using the Nanodrop®ND100 UV-Vis spectrophotometer (NanoDrop Technologies Inc., Wilmington, Del., U.S.A.), as well as by visualization on an Agilent 2100 bioanalyzer (Agilent Technologies Inc., Santa Clara, Calif., U.S.A.). RNA samples underwent *DNase I* treatment using the Ambion RiboPure™ Bacteria Kit (Ambion) following the manufacturer's instruction with 1 modification: the samples were incubated for 2.5 h at 37 °C to sufficiently digest the genomic DNA. Samples were requantitated using the Nanodrop®ND100 UV-Vis spectrophotometer after *DNase I* treatment. *DNase I*-treated RNA (90 to 250 ng) was used for cDNA synthesis. Reactions with and without reverse transcriptase were also performed as negative controls. Synthesis of cDNA was performed using the SuperScript III First-Strand Synthesis SuperMix Kit (Invitrogen, Carlsbad, Calif., U.S.A.) following the manufacturer's instruction.

### Primer design and real-time PCR

Twenty-seven genes related to *B. anthracis* sporulation and germination were chosen from among 31 genes presented by Day and others (2007). The primers were designed using Primer 3 (version 0.4.0) software (http://frodo.wi.mit.edu). Primer sequences were blasted using the NCBI blastn program against the nonredundant (nr) database, which indicated that the primer sequences showed homology to the *B. anthracis* Ames strain (GenBank accession nr AE017334). Primers were synthesized and purchased from IDT (www.idtdna.com). Primers for the housekeeping genes *16srRNA, gyrase B, spo G, Adh, and rpoB* were tested, in which *spoG* showed the most consistent *Ct* values and was chosen as the internal control gene for quantification.

PCR was performed in a 96-well plate on a Bio-Rad iQ5 real-time PCR system (Bio-Rad, Hercules, Calif., U.S.A.) in a 25-$\mu$L total volume, and contained 2.5 $\mu$L of 10× PCR Buffer (−$MgCl_2$), 1.5 $\mu$L 50 mM $MgCl_2$, 0.5 $\mu$L 10 mM dNTP mix, 3.75 $\mu$L units Taq DNA Polymerase, recombinant (all Invitrogen), 0.3 $\mu$L 20× EvaGreen (Biotium, Hayward, Calif., U.S.A.), 1.25 $\mu$L of each primer at 10 $\mu$M, varying amounts of cDNA (see previously mentioned under cDNA synthesis) and nuclease-free water (Ambion). Amplification involved an initial denaturation at 95 °C, followed by 40 cycles of 95 °C for 15 s, 59 °C for 25 s, and 72 °C for 30 s. Fluorescence data were collected at the 59 °C annealing step. The final step was a melt curve of 81 cycles with a temperature range of 55–95 °C for 30 s with an increased set-point temperature after cycle 2 by 0.5 °C. Results were visualized using the iQ5 version 2.0 software provided with the thermocylcer. To determine relative gene expression, the value of the internal control gene (*spoG*) was subtracted from the treated and untreated samples. The $\Delta Ct$, $\Delta\Delta Ct$, and the $2^{-fx}$ values were calculated as described (Pfaffl 2001). All PCR experiments were performed in triplicate and the averaged *Ct* values from 3 independent experiments were used for calculation.

## Results and Discussion

Twenty-seven sporulation- and germination-related genes identified by the TraSH assay (Day and others 2007) were analyzed for expression using real-time PCR following various intervention strategies. These genes have been shown to be related to *B. anthracis* Sterne sporulation and germination using microarray assays (Day and others 2007). RNA isolated from different treatments was reverse transcribed to cDNA; primers designed from these genes (Table 1) were used to perform real-time PCR assays. Compared to the values of the untreated samples, the 2-fold increase and decrease (>2-fold and <0.5-fold on Table 2) were chosen as the cut-off values for further discussion.

### Heat treatment

To study how heat treatment affects the bacterial spores, RNA from heat-treated (80 °C for 10 min) and nonheat-treated spores was reverse transcribed and subject to real-time PCR analysis. As shown in Table 2, the expression levels of 5 of the 27 target genes were elevated by at least 2-fold after the heat treatment. Of these 5 genes, genes that encode for an ABC transporter, GTP pyrophosphokinase, germination protein gerN, and spore germination protein GerHC are related to spore germination (Leskelä and others 1996; Weiner and others 2003; Ryu and others 2007). Interestingly, the expression level of the gene encoding for stage II sporulation protein P was elevated by more than 8-fold. On the other hand, the expression levels of genes encoding spore germination operon were also reduced by more than 2-fold, whereas some sporulation-related genes were also reduced more than 2-fold (<0.5 on Table 2). Our results indicate that heat treatment of spores affects the expression levels of both sporulation and germination related genes.

### Pasteurization

To study whether the standard pasteurization procedure affects sporulation and germination, real-time PCR was performed using the RNA before and after pasteurization. Of the 27 genes we tested, there was no gene that showed either a 2-fold activation or inhibition (Table 2). This indicates that at the molecular level, the expression levels of the twenty-seven genes related to sporulation and germination were not appreciably affected by pasteurization. Previous findings indicate that pasteurization did not kill *Bacillus* spores (Bowen and Turnbull 1992; Perdue and others 2003; Novak and others 2005). Consistent with this finding, our data further support that pasteurization did not have any major influence on spore sporulation and germination. However, only a small set of genes related to sporulation and germination were tested in this study. For a more comprehensive study, a high-throughput assay, such as a microarray assay, may be needed.

## Microfiltration and microfiltration and pasteurization

Microfiltration removes bacterial spores effectively from milk (Elwell and Barbano 2006). In addition, the combination of microfiltration and pasteurization had an additive effect on bacterial removal (Elwell and Barbano 2006). To investigate whether microfiltration or the combination of microfiltration and pasteurization affects sporulation or germination, RNA from samples under these treatments at different times (30 and 120 min after microfiltration treatment) and the samples before these treatments were used to perform real-time PCR assay. As shown in Table 2, 7 genes were induced in the R30 (retentate in 30 min after microfiltration treatment). Of the 7 genes, only an ABC transporter gene is related to spore germination, the other 6 genes are all sporulation related. Interestingly, genes encoding for stage II sporulation proteins E and A, and stage IV sporulation protein A were induced in both R30 and R120 (retentate in 120 min after microfiltration treatment). None of the genes tested were inhibited by microfiltration. On the other hand, no genes were significantly induced or inhibited by the combination of pasteurization and microfiltration treatment in both PR30 and PR120 (retentates in 30 and 120 min after pasteurization and microfiltration treatment). Our data suggest that microfiltration alone may induce some sporulation genes whereas the combination of microfiltration and pasteurization did not result in major expression changes in sporulation- and germination-related genes.

**Table 1 — Genes and primer sequences used for real-time PCR analysis.[a]**

| Gene | Primer sequences | Product size (bp) | Description |
|---|---|---|---|
| BA1048 | tattttagcgacggcagaac<br>cgcctggcatattaaactgt | 113 | ABC transporter, ATP-binding protein EcsA |
| BA4151 | tgctactgctacaaatgcaact<br>ggcgataaagcaaacgaata | 136 | cytochrome c oxidase, subunit IVB |
| BA5050 | aaaccgaaaaggaaaactgg<br>tccatggttgacgacctact | 125 | cytochrome d ubiquinol oxidase, subunit I |
| BA4637 | tgcacgtttacgcttcatta<br>ttggcacatagactcggaat | 117 | GTP pyrophosphokinase, RelA |
| BA1510 | aaatgcaccgaaagtacagc<br>caattacgaccattccttgc | 131 | negative regulator of competence MecA, putative |
| BA0424 | gtgccagcaactttctttgt<br>gtgactccacgaatggtttc | 108 | polysaccharide deacetylase, PdaA |
| BA1639 | cgcccagcatagttgtactt<br>tggtcttgttatgggaatgg | 140 | germination protein GerN |
| BA4986 | cgtcttgatgcacgtattca<br>aggatcgacgcctaaagatt | 150 | spore germination protein GerHC |
| BA0634 | tcctatagaagcagcgcagt<br>ttccagaaccagtcgcttac | 137 | spore germination protein GerKB |
| BA0709 | tggagcaagagaaggtgttc<br>gtttcggcaacctaattcct | 101 | spore germination protein GerLA |
| BA0710 | cagctttccaaacggaagta<br>gtgccttttttcgctgtctta | 126 | spore germination protein GerLB |
| BA0763 | ctcaccgtcacgtttttctt<br>tatacccatttcgcggttta | 143 | spore germination protein GerYA |
| BA1492 | aattcatgtcccagcagaaa<br>cttgcatcgtagaagccaat | 140 | spore maturation protein |
| BA4546 | agaggcaacttcatcaatcg<br>tgctgtagaggcacatcaaa | 133 | spore protease |
| BA5730 | acctgcaatcacttcctctg<br>ttgagtgaagcaccaagtca | 102 | sporulation initiation inhibitor protein Soj |
| BA2644 | tattaacgacgcttcggatt<br>tcgatttggtgaaccgtatt | 119 | sporulation kinase B |
| BA4530 | tcggactttcctcattacca<br>gccgcaaaatttagtagcaa | 143 | sporulation protein |
| BA4394 | atatgagcaggcacaccaat<br>cggtaaggcaaacgctacta | 132 | stage 0 sporulation protein A |
| BA5581 | ctcatttctcaccgcttgac<br>gactgcttatggtgagcttga | 112 | stage 0 sporulation protein F |
| BA4688 | gcctccattacctttccact<br>aacacaagcaccaaaagagg | 145 | stage II sporulation protein B, putative |
| BA0061 | ttaagcctggcaaatgtctc<br>Tagcgtaatcaggcttgtcc | 138 | stage II sporulation protein E |
| BA2068 | aagaacggactggagaaggt<br>ttcccaactatgcgaatgat | 122 | stage II sporulation protein P |
| BA1530 | gaaccaattccattccatga<br>tttcaccaatcgttccatct | 106 | stage IV sporulation protein A |
| BA4396 | attgccaatcacttcacgat<br>ggacaaattatgcgttcgac | 105 | stage IV sporulation protein B |
| BA4643 | acaccgcttacaacaagctc<br>gtgcaagaagctggatgaat | 140 | stage V sporulation protein B |
| BA0054 | ggctgaaagtgcaaaagtgt<br>ggaataagcgacaacgagaa | 102 | stage V sporulation protein B, putative |
| BA4050 | tccaattgcgagtaacgatt<br>atgtttggtttgcttggtgt | 147 | stage V sporulation protein E |

[a]Gene descriptions were obtained from The Inst. for Genomic Research genome annotation (Read and others 2003).

**Table 2— Relative expression levels of sporulation and germination-related genes under different treatments in *B. anthracis* Sterne strain.**

| Gene description | Treatments | | | | | |
|---|---|---|---|---|---|---|
| | P[a] | R30[b] | R120[c] | PR30[d] | PR120[e] | heat |
| ABC transporter, ATP-binding protein EcsA | 1.2 | 2.4 | 1.2 | 1.2 | 1.3 | 2.3 |
| Cytochrome c oxidase, subunit IVB | 0.9 | 1.6 | 1.1 | 1.0 | 1.2 | 1.8 |
| Cytochrome d ubiquinol oxidase, subunit I | 0.9 | 1.4 | 1.2 | 0.9 | 1.2 | 0.3 |
| GTP pyrophosphokinase | 1.1 | 1.4 | 1.1 | 0.9 | 1.2 | 2.1 |
| Negative regulator of competence MecA, putative | 0.6 | 1.6 | 1.1 | 0.9 | 1.1 | 0.1 |
| Polysaccharide deacetylase, putative | 0.9 | 1.5 | 1.1 | 1.4 | 1.3 | 0.1 |
| Germination protein gerN | 0.6 | 1.6 | 1.3 | 0.9 | 1.2 | 4.4 |
| Spore germination protein GerHC | 1.3 | 1.5 | 1.1 | 0.9 | 1.1 | 2.5 |
| Spore germination protein GerKB | 0.6 | 1.4 | 1.1 | 1.0 | 1.2 | 0.3 |
| Spore germination protein GerLA | 0.6 | 1.9 | 1.0 | 1.0 | 1.2 | 0.4 |
| Spore germination protein GerLB | 0.7 | 1.5 | 1.1 | 0.9 | 1.2 | 0.3 |
| Spore germination protein GerYA | 0.7 | 1.6 | 1.0 | 1.0 | 1.2 | 0.1 |
| Spore maturation protein | 0.7 | 1.4 | 1.0 | 0.9 | 1.2 | 0.8 |
| Germination protease precursor | 0.6 | 1.4 | 1.0 | 0.9 | 1.0 | 0.4 |
| Sporulation initiation inhibitor protein Soj | 0.6 | 1.4 | 1.1 | 1.0 | 1.2 | 1.3 |
| Sporulation kinase B | 1.1 | 1.6 | 1.1 | 1.6 | 1.6 | 0.7 |
| Sporulation protein | 0.9 | 1.7 | 1.1 | 1.0 | 1.2 | 0.7 |
| Stage 0 sporulation protein A | 0.6 | 1.4 | 1.0 | 1.1 | 1.0 | 1.3 |
| Stage 0 sporulation protein F | 0.8 | 1.3 | 0.9 | 0.9 | 1.0 | 1.3 |
| Stage II sporulation protein B, putative | 0.5 | 1.7 | 0.8 | 1.0 | 1.3 | 0.5 |
| Stage II sporulation protein E | 0.9 | 2.8 | 2.0 | 1.2 | 1.6 | 0.3 |
| Stage II sporulation protein P | 0.5 | 2.9 | 2.2 | 0.8 | 1.1 | 8.6 |
| Stage IV sporulation protein A | 1.2 | 3.5 | 2.5 | 1.4 | 1.7 | 0.4 |
| Stage IV sporulation protein B | 0.6 | 2.0 | 1.3 | 1.1 | 1.0 | 0.7 |
| Stage V sporulation protein B | 0.6 | 2.2 | 1.4 | 0.8 | 1.1 | 0.2 |
| Stage V sporulation protein B, putative | 0.6 | 2.2 | 1.5 | 1.0 | 1.1 | 1.2 |
| Stage V sporulation protein E | 0.7 | 2.7 | 1.5 | 0.9 | 1.4 | 1.5 |

All PCR experiments were performed in triplicates and the averaged *Ct* values from 3 independent experiments were used for calculation. The numbers (fold change) were calculated using the untreated samples as 1.
[a]P stands for pasteurization (72 °C for 15 s).
[b]R30 stands for 30 min in retentate after microfiltration treatment.
[c]R120 stands for 120 min in retentate after microfiltration treatment.
[d]PR30 stands for 30 min in retentate after pasteurization and microfiltration treatments.
[e]PR120 stands for 120 min in retentate after pasteurization and microfiltration treatments.



## Conclusions

In our study, twenty-seven germination- and sporulation-related genes were tested using real-time reverse transcriptase PCR assays under different intervention strategies. Our data demonstrate that there was no common gene that was induced or inhibited by all of the intervention strategies tested. Heat treatment both induced and inhibited sporulation- and germination-related genes. Microfiltration treatment only resulted in gene activation but not inhibition. Finally, pasteurization and pasteurization and microfiltration did not result in any major changes of tested sporulation- and germination-related genes.

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